IJPSR (2012), Vol. 3, Issue 09 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 24 April, 2012; received in revised form 27 June, 2012; accepted 19 August, 2012

IN VITRO AND *IN VIVO* EVALUATION OF POLY (CAPROLACTONE FUMARATE) NANOPARTICLES

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Keywords:
Poly (caprolactone fumarate),
Doxorubicin HCl,
Cytotoxicity,
Cellular uptake,
In vivo imaging

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ABSTRACT

Poly (caprolactone fumarate) (PCLF) copolymer has been synthesized by polycondensation of biodegradable, biocompatible and FDA approved poly (caprolactone) and fumaryl chloride and has been used for tissue engineering. In this study, ligand-less PCLF nanoparticles (NPs) were evaluated as a drug delivery system (DDS) with a potential of improved lymphoma tissue uptake regarding PCLF hydrophobicity. Doxorubicin HCl (Dox) release profiles showed a burst release of 27% during initial 6 hours following by a desired controlled release of 85% in 4 days in pH 5.8 (tumor pH). Empty PCLF NPs did not cause a considerable cytotoxicity on T47D, HT29 and 3T3NIH model cell lines, indicating the polymer safety. Cytotoxicities of Dox loaded PCLF NPs were almost equal to those of Dox alone revealing the NPs ability for tumor treatment. Fluorescent microscopy images proved an efficient uptake of PCLF NPs by T47D model cells. *In vivo* images showed two days accumulation of PCLF NPs in liver and spleen and specially in lymph node rich regions of mouse body after 24 hours of injection. Such characteristics made PCLF NPs DDS a promising candidate for treatment of lymphoma or other lymphatic disorders.

INTRODUCTION: Among different kinds of nanoparticles (NPs), polymeric NPs are promising vehicles for drug delivery because of their high stability, drug loading, controlled release and drug protection against environmental effects and also their easier manipulation for different purposes [1]. To reduce anticancer wide side effects, tremendous works have been done on specifically delivering anticancers to the tumor tissues by active (ligand-mediated) or passive (ligand-less) targeting. Since tumor vasculature are porous and their drainage is low (Enhanced Permeation and Retention, EPR, effect) and since NPs have prolonged blood circulation time, NPs with 100-200 nm size can enter and be entrapped and therefore be passive targeted into tumor tissues [2, 3].

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Nevertheless, despite the wide researches in the development of NPs, studies on their potential toxicity, needs more attention [4]. Similarly, the characteristic biokinetic behavior of NPs is an attractive quality for promising applications in medicine. Their biological activity and biokinetics are dependent on many parameters: size, shape, chemistry, charge, surface modifications, etc. When NPs be ingested, systemic uptake of NPs via lymph can occur and they can distribute and be taken up by liver, spleen, bone marrow, heart or other organs. Due to the ethical concerns and also reliable *in vivo* experiments, in the last few decades new technologies such as whole animal *in vivo* imaging have been developed [5].

In vivo fluorescence imaging uses a sensitive camera to detect fluorescence emission from fluorophores in whole-body living small animals. To overcome the photon attenuation in living tissue, fluorophores with long emission at the near-infrared (NIR) region are generally preferred, including widely used small indocarbocyanine dyes [6, 7] such as the recently developed NIR, lipophilic carbocyanine dye, 1, 1′-dioctadecyl- 3, 3, 3′, 3′-tetramethylindotricarbocyanine iodide (DiR). DiR has been used to safely and directly label the membranes of human leukemic cell lines, primary murine lymphocytes and erythrocytes [8] as well as NPs for detection in live animals [9-11] and is also approved for clinical use in hepatic function examination and fluorescent angiography [12].

Poly (caprolactone), (PCL), is a biodegradable, bio-compatible and FDA approved polymer synthesized through ring opening polymerization of caprolactone. PCL has been used for bioresorbable sutures, scaffolds and micro and nanoparticulate drug delivery systems [13, 14]. Several copolymers (polyesters) have been synthesized from PCL and a hydrophilic segment, degrading by esteric hydrolysis [15, 16]. Such a copolymer is Poly (caprolactone)-poly (ethylene glycol) used for preparation of nanoparticulate drug delivery systems [17,18].

In our previous works [19, 20], synthesis and identification of poly(caprolactone fumarate) (PCLF) copolymers were performed according to Wang *et al*., (21-24) by polycondensation of fumaryl chloride and PCLdiols with nominal Mn of 530, 1250 and 2000 g mol^{-1}, resulting in PCLFs named as PCLF530, PCLF1250 and PCLF2000 (Mw of 6026, 9287 and 11623 g/mol, respectively). Moreover, PCLF NPs with average size of 225 nm, zeta potential of about -40 mV and doxorubicin HCl (Dox) loading of 6.8%, were prepared and characterized [19]. In addition, in a separate work, crosslinking of the NPs via fumarate double bonds [25, 26] was investigated [20]. Also it was found that PCLF was an easily tunable copolymer, for example by using different Mw of PCLdiol, and therefore, it can be designed for preparation of delivery systems with different purposes.

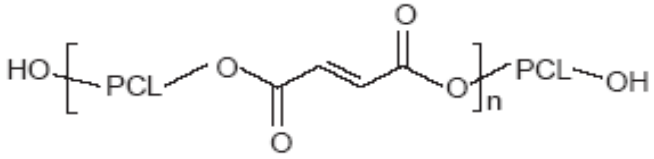

FIGURE 1: SCHEME OF PCLF

Since macrophages in reticuloendothelial system tend to phagocyte more hydrophobic molecules, we were hoping that the hydrophobicity of PCLF NPs can enhance their lymphatic uptake and be useful for lymphoma therapy via passive targeting. When lymphoma occurs, cells in the lymphatic system divide too rapidly. Because there is lymph tissue in many parts of the body, the cancer cells may involve the liver, spleen, or bone marrow [27].

In this study, the cytotoxicity effect of the PCLF NPs on cancerous and normal cells, the ability of cancerous cells to uptake PCLF NPs and *in vivo* distribution of these NPs in live mice were evaluated. Since we have proved that NPs of PCLF1250 not only possessed a lower size than NPs of PCLF2000, but also could load more drug than NPs of PCLF530, cell culture tests were more focused on the NPs of PCLF1250. Besides, NPs of PCLF2000 were used for *in vivo* experiments because of their higher hydrophobicity than two other kinds of NPs.

MATERIALS AND METHODS:

Materials: Dox, fluorescein isothiocyanate (FITC) and MTT were purchased from sigma-aldrich Company. Acetone and other chemicals were purchased from Merck, Germany and all were of synthesis grade. RPMI 1640 medium, FBS and Trypsin-EDTA were purchased from Gibco company, and DiR was purchased from Bioquest company.

Preparation of PCLF NPs: The preparation method of PCLF NPs and the method of Dox release test were reported previously [19]. Briefly, acetone containing PCLF (0.6 w/v%) was poured into deionized water containing Dox (0.1w/v%), to obtain Dox loaded NPs. In this paper, the Dox release profile in phosphate buffered saline, PBS, pH 5.8 (indicating tumor pH) was obtained to compare to that in PBS pH 7.4.

***In vitro* cytotoxicity test:** The cytotoxicity of PCLF NPs was investigated on model cell lines of T47D (breast cancer cells), HT29 (colorectal carcinoma cells) and NIH3T3 (normal fibroblast cells) using the colourimetric MTT *in vitro* assay. Complete RPMI 1640 culture medium (Dulbecco's modified essential medium, Gibco co.), supplemented with 5% (v/v) fetal bovine serum (Gibco co.), and 1% (v/v) antibiotic (2 mM L-glutamine, 100 U/mL Penicillin and 0.1 mg/mL Streptomycin; Gibco) was used. The cryotube content was added to 1 ml of the medium in a sterile 15 ml falcon tube to diminish the effect of DMSO following centrifugation at 2000 rpm to obtain cell pellet.

Then, 5 ml of the medium was added and transferred to a T_{25} flask under a sterile condition. Cultured cells were kept at 37˚C in a humidified 5% CO2 incubator. Once the cells reached confluence, the culture medium was removed and the cells were rinsed three times with sterile PBS (5ml/25cm) to remove the serum. The confluent cell layers were enzymatically removed, using 2.5 ml of trypsin/EDTA (Gibco, USA). The mixture was centrifuged at 2000 rpm in 25˚C for 5 minutes. 2 ml of the medium was added to the precipitated cells (cell pellet).

Cell viability was assessed by indirect cell count, MTT (3-[4,5-dimethylthiazol-2-yl]-2-5diphenyltetrazolium bromide) method: MTT solution in buffer converts to the insoluble formazan with a purple color in mitochondria of the live cells as the effect of dehydrogenase enzymes which are existed in MTT. Death cells can not convert the MTT to formazan, therefore, the level of produced formazan is related to the vital cells. Overall, samples, 25 µl of MTT solution (4mg/ml PBS), 10^4 cell/well and 180 µl medium were added to every well of a 96-well plate, following by incubation at 37˚C and removing the medium containing MTT after 48 hours. Then, 100 µl of DMSO was added to every well to dissolve the formazan crystals. The ratio of added NP suspension to the medium was 0.5ml/1.5ml. A colorimetric assay measured the level of the dye and the results, light absorbances, were determined with a plate reader in 540 nm (OD_{540}) (ELISA reader: absorbance of surviving cells). Each experiment was repeated on three separate occasions. Two internal controls were set up for each experiment:

1. An IC_{50} consisting consisting the dose killing 50% of cells, and;

2. IC_{100} consisting of medium only.

Background absorbance due to the non-specific reaction between test compounds and the MTT reagent was deducted from exposed cell values. Viability percentage compared to a control without treatment was caculated as:

$$\text{Viability (\%)} = Ni/Nc \times 100$$

Viability %: The percentage of mean number of vital cells, Ni: mean light absorbance of three wells of samples, Nc: mean light absorbance of three wells of controls.

Dose response curves of *in vitro* cytotoxicity data were obtained. Dose response curves were plotted for the test chemicals after correction by subtracting the background absorbance from the controls. The sample formulations were as follow:

Sample 1: Suspensions of PCLF530 NPs in RPMI with the NP concentration of 6000, 3000 and 1500 µg/ml,

Sample 2: Suspensions of PCLF1250 NPs in RPMI with the NP concentration of 6000, 3000 and 1500 µg/ml,

Sample 3: Suspensions of PCLF2000 NPs in RPMI with the NP concentration of 6000, 3000 and 1500 µg/ml,

Sample 4: Suspension of Dox loaded PCLF1250 NPs in RPMI with the NP concentration of 6000, 3000 and 1500 µg/ml and the Dox concentration of 60, 30 and 15 µg/ml, respectively,

Sample 5: Solutions of Dox in RPMI (as positive control) with concentrations of 100 and 250 nM for T47D cells and 300 and 500 nM for HT29 cells.

Cellular Uptake Test: First, PCLF NPs physically encapsulating FITC, were prepared with average size of about 200 nm and negative zeta potential. To observe uptake of NPs, 100,000 cells were placed in every well of a 24-well multiwell plate and incubated for 48 hours. Then, the samples were added to every well and incubated for 48 hours. After that the cells were imaged with confocal microscopy (IX71, Olympus, Japan) immediately after replacing the particle suspension in the medium with fresh particle-free medium, to observe the uptake of PCLF NPs. Samples used were as follow: Sample 1: A suspension of PCLF1250 NPs in RPMI with the NP concentration of 6000 µg/ml, Sample 2: A suspension of FITC loaded PCLF1250 NPs in RPMI with the NP concentration of 6000 µg/ml and FITC concentration of 0.25 µg/ml.

***In vivo* imaging**: First, DiR excitation and emission wavenumbers were determined by *in vivo* imaging system (Kodak, USA). Then DiR was loaded into PCLF NPs by dissolving it in acetone phase during NP preparation process. The NPs were isolated by a paper filter (PC type, Whatman, UK) with pore size of 0.1 µm and the filtrate UV-VS absorbance was read at 741.5 nm (Jasco V-530, Jasco co., Japan) for determination of the concentration of unloaded DiR. The latter amount was subtracted from the initially added DiR to acetone before the production of NPs and the result was the amount of DiR loaded into the NPs. Ultimately, this amount of loaded DiR was used to calculate the drug loading (DL) and entrapment efficiency (EE) by the following equations:

DL (%) = Amount of drug in NPs / Amount of drug-loaded NPs × 100

EE (%) = Amount of drug in NPs / Total amount of drug × 100

The profiles of DiR release from PCLF NPs were obtained in PBS pH 7.4 as described in the paragraph of PCLF NPs. A whole animal imaging was performed on laboratory BALB/c mice after itracardial injection of 20 µl DiR loaded PCLF NPs (formulations listed in **table 1**, passed through a 0.45 µm sterile filter) or DiR solution or saline as controls. Anesthesia was delivered before every imaging by subcutaneous injection of ketamine hydrochloride at dose of 10 mg/kg and xylazine at dose of 1 mg/kg.

TABLE 1: DiR LOADING INTO PCLF NPs

F.	PCLF Mw (w/v%)	DiR (w/v%)	NP size (nm)	PDI	Zeta potential (mV)	DL%	EE%
1	530,0.6	0.2	126±20.4	0.1	-21.6±7.5	7.6±4	46±24.2
2	1250,0.6	0.2	270.6±10.2	0.1	-3.3±2	21.6±7	65±21
3	2000,0.6	0.2	247±6.2	0.1	-9±2	30.6±0.5	92±1.7
4	PCL2000,0.6	0.2	302.6±34.2	0.2	0.6±0.4	20.3±18.5	61±25.5

Statistical Analysis: The data were presented as mean ± SD. One-way analysis of variance (ANOVA) was used for comparing the mean differences. SPSS for Windows (release 11.5.0) was employed for statistical analysis. p-value <0.05 was considered to be significant. In this study, all of the experiments were performed in triplicate, and the averages were considered to be the response.

RESULTS AND DISCUSSION:

Dox Release Profile: The profile of Dox release in PBS pH 5.8 indicating the pH of the tumors, was characterized by an initial burst release of 27% in first 6 hours following by a sustained release of about 85% after 4 days to 90% after 7 days, while previously reported profile in pH 7.4 contained a constant maximum release of about 80% after 4 days (**figure 2**).

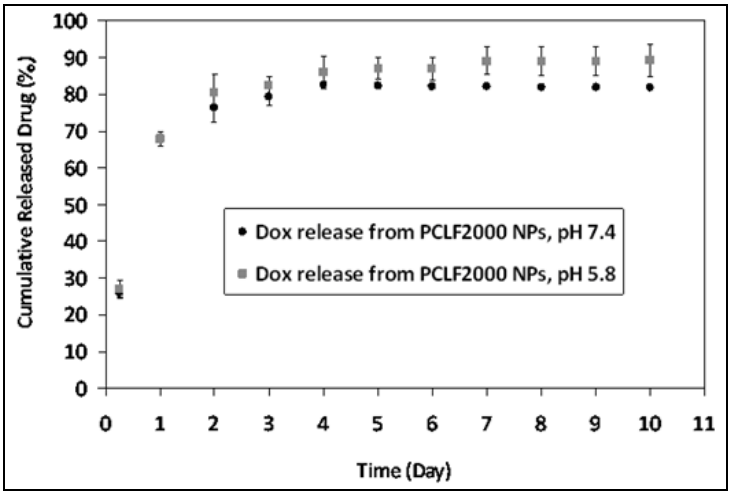

FIGURE 2: PROFILES OF RELEASE OF TRB AND DOX FROM FORMULATIONS 15 AND 21 (TABLE 5), RESPECTIVELY, IN PBS (PH 7.4) AND AT 37˚C. Data are shown as cumulative percent of released drug and as mean ± SD (n=3)

***In vitro* Cytotoxicity**: **Figures 3, 4 and 5** show the cell viability (%) after exposuring them to the different PCLF NPs or Dox solutions. Each experimental rectangle represents the average of a series of three different experiments. Cell viability was about 90% after T47D, HT29 and NIH3T3 cells were exposured by non-loaded PCLF NPs, indicating the safety of the polymer for the cells. There were no significant different between cell viability for PCLF530, 1250 and 2000 NPs in all three cell lines ($p>0.05$). Obviously, cell viability was significantly reduced in a dose-dependent manner after exposuring the cells to Dox solutions or Dox loaded NPs ($p<0.05$).

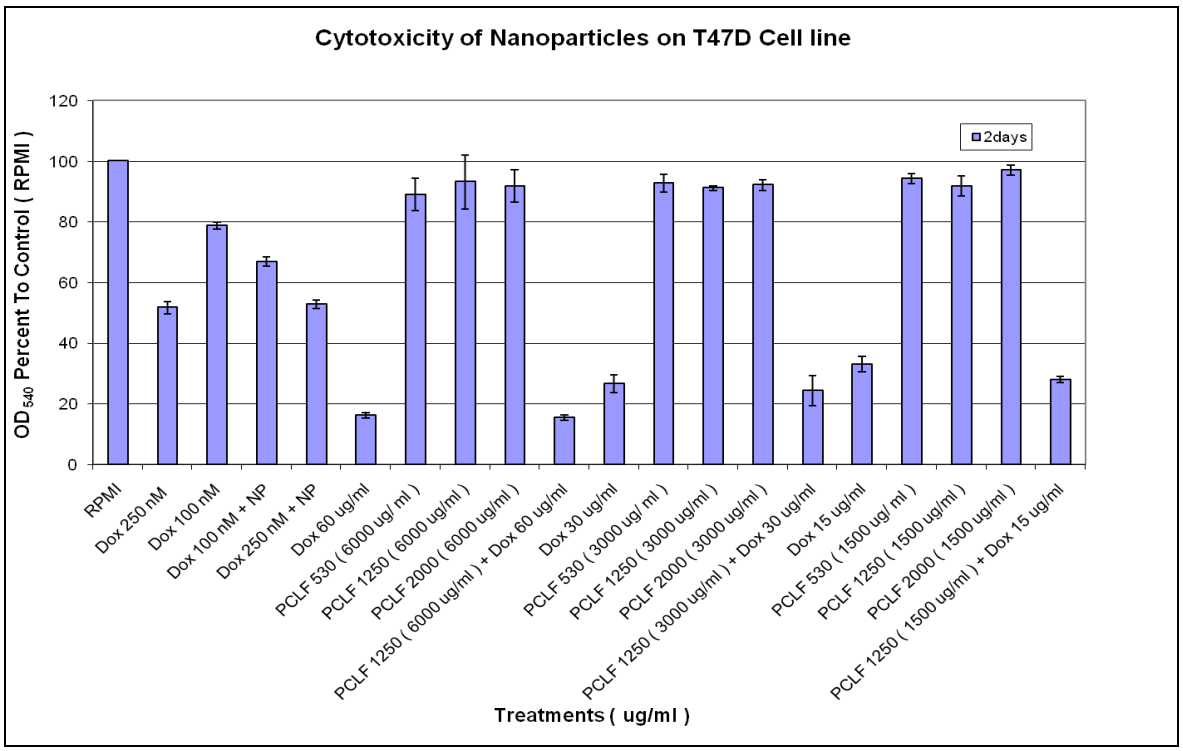

FIGURE 3: CELL VIABILITY OF PCLF NPs AFTER 24 H EXPOSURE TO T47D CELLS

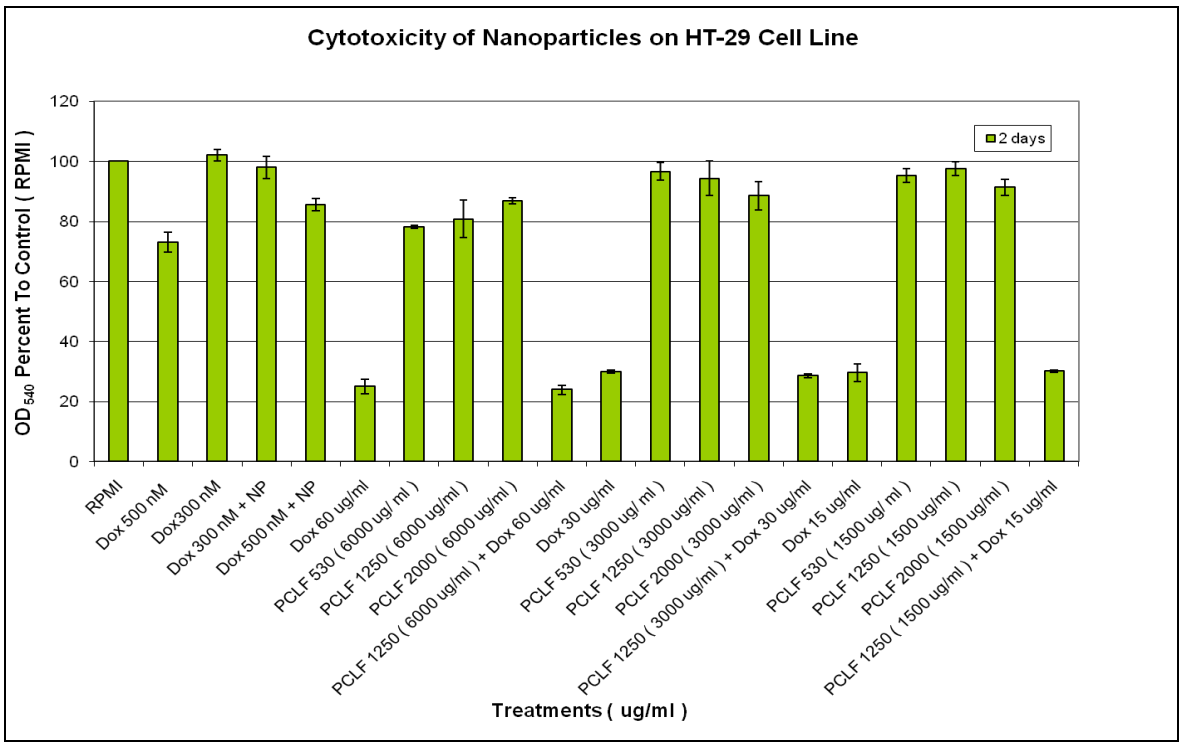

FIGURE 4: CELL VIABILITY OF PCLF NPs AFTER 24 H EXPOSURE TO HT29 CELLS

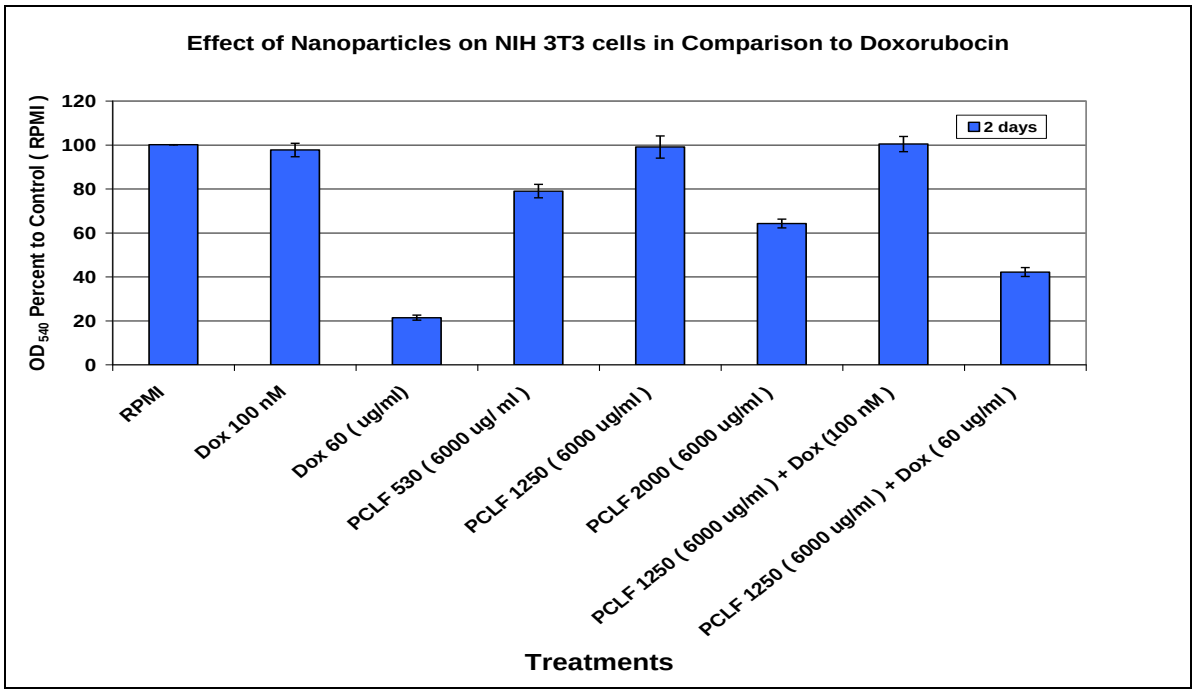

FIGURE 5: CELL VIABILITY OF PCLF NPs AFTER 24 H EXPOSURE TO NIH3T3 CELLS

By increasing the Dox concentration, the cytotoxicity reduced dramatically ($p<0.05$). Since the IC50 for T47D cells was lower than that for more resistant HT29 cells, they showed higher viability compared to T47D cells. Exposuring the cells to the Dox loaded NPs, indicated a range of cytotoxicity responses which were almost equal to the cytotoxicity caused by Dox solutions.

Since Dox release from NPs needed three days, the latter observation could prove an effective uptake of NPs by the cells. Results of this study demonstrated that the MTT assay could be implemented as an effective and sensitive tool to assess cytotoxicity of PCLF NPs on cells.

Cellular uptake of NPs: Consistent with the results of confocal microscopy (**Figure 6**), fluorescence signals were detected in the T47D cells after 48 hours incubation with FITC loaded PCLF NPs**.** Figure 6, A, shows T47D cells incubated with PCLF1250 NPs in RPMI medium. As there was no fluorescent agent in the formulation, naturally no fluorescence color was observed in the image. Figure 6, B, the image of incubation of the T47D cells with FITC loaded PCLF1250 NPs, showed green fluorescence inside the cells, especially in the perinuclear region, revealing the cellular uptake of the NPs. As the intense fluorescence color in the image represents, such NP uptake by T47D cell line was predominant.

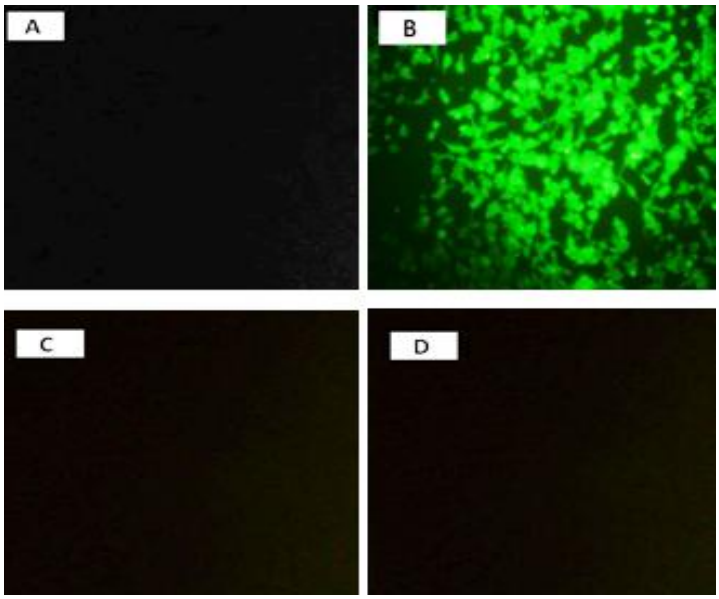

FIGURE 6: IMAGES FROM FLUORESCENT MICROSCOPE TO INVESTIGATE THE POSSIBILITY OF PCLF NPs CELLULAR UPTAKE; A: T47D cells exposured by PCLF NPs, B: T47D cells exposured by FITC loaded PCLF NPs, C: T47D cells exposured by RPMI alone, D: T47D cells exposured by RPMI and acetone

Figure 6, C and D, were controls showing the cells incubated with RPMI alone and RPMI with acetone, respectively. These images demonstrate that the RPMI medium and the acetone (which can be a residue of the NP preparation process) could not make any fluorescence signal in the image. Therefore, the intracellular fluorescence of T47D cells (Figure 6, A), proved the ability of these cells to uptake the PCLF NPs.

***In vivo* distribution of NPs:** Experiments showed that DiR excitation and emission was at 670 and 790 nm, respectively. This allows us to obtain a significant signal with very low background level and therefore a high resolution (8). Table 1 shows the values of DiR loading into the PCLF NPs and **figure 7** shows DiR release profiles. DiR was released from PCLF530 NPs in 4 days (70%) and from PCLF 1250, 2000 and PCL NPs in 6 days (60, 60 and 80%, respectively).

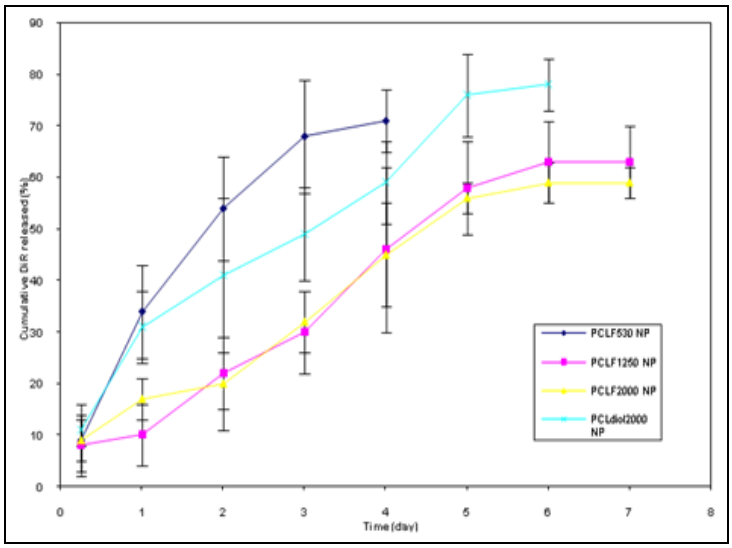

FIGURE 7: DiR RELEASE FROM PCLF NPs

Therefore, fortunately most of the DiR was remained in the NPs and not released during the 3 days of *in vivo* imaging experiment.

The distribution of DiR solution and DiR loaded NPs were explored over three days following intracardial injections to mice (which have similar size of lymphatic capillaries as those in humans-10-80 µm). According to the *in vivo* images (**Figures 8-11**) it was found that both DiR and NPs entered the liver after 30 minutes but the NPs continued to enter and remain in other organs such as spleen and axillary and terminal lymph nodes, while DiR excreted slowly from the body after few hours.

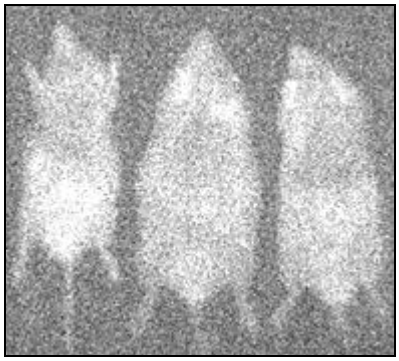

FIGURE 8: NOT INJECTED MICE

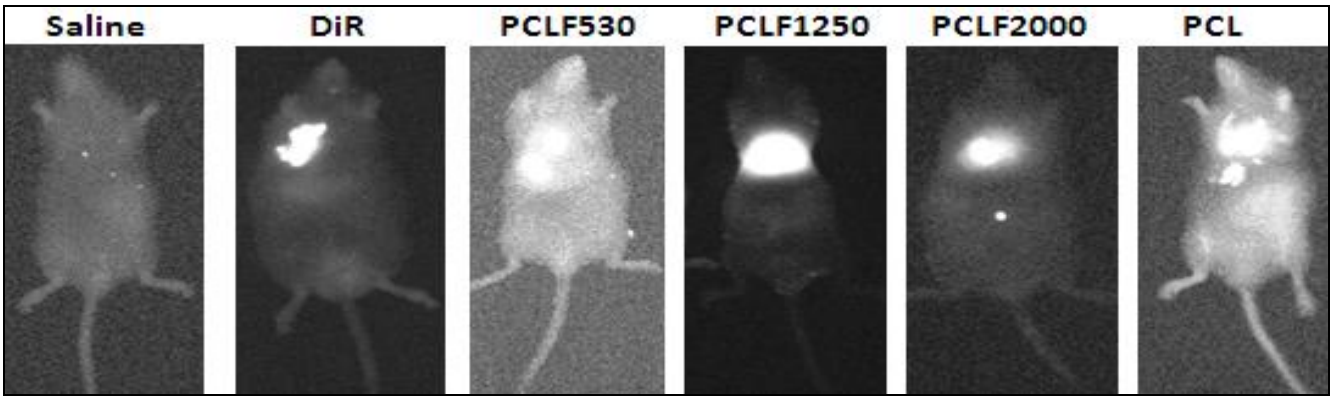

FIGURE 9: IMAGES AT TIME: 0 (AFTER INJECTION)

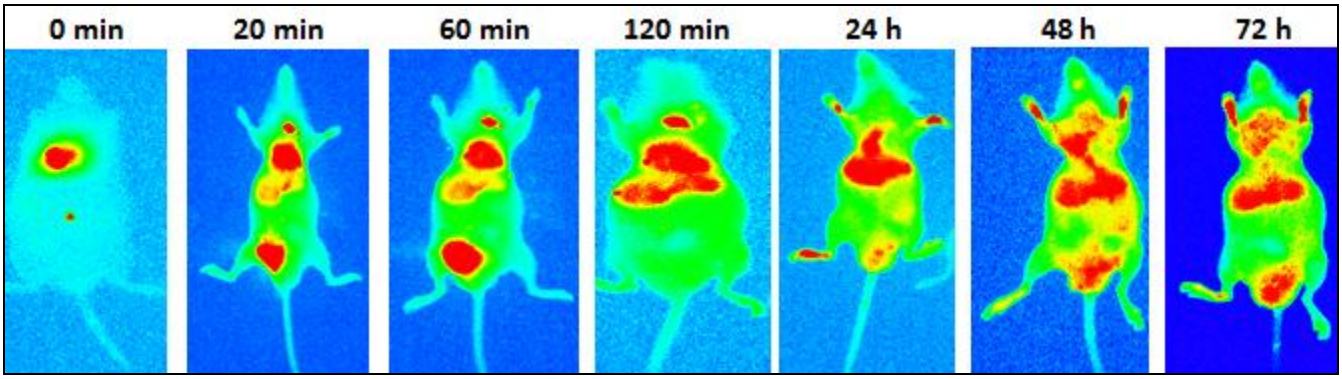

FIGURE 10: DISTRIBUTION OF PCLF2000 NPs

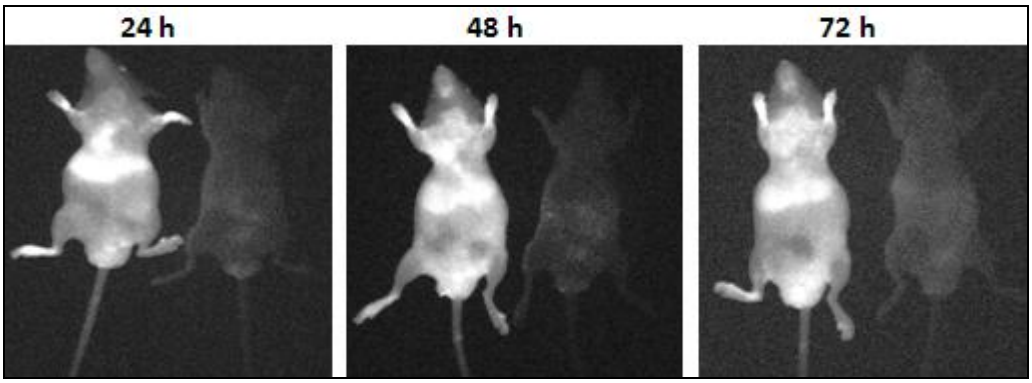

FIGURE 11: COMPARISON OF PCLF2000 NPs AND DiR

It can be concluded that the NPs were taken up into the lymphatic nodes and that they were retained in the lymph nodes for two days. The ability of such ligand-less NPs to deliver drugs to macrophages in lymph nodes, offers a promising approach to immunotherapy and lymphoma therapy [7].

Lymph node retention time of NPs has been investigated by several other researchers and typically has focused on time points in the range of 6-52 hours post-injection [8].

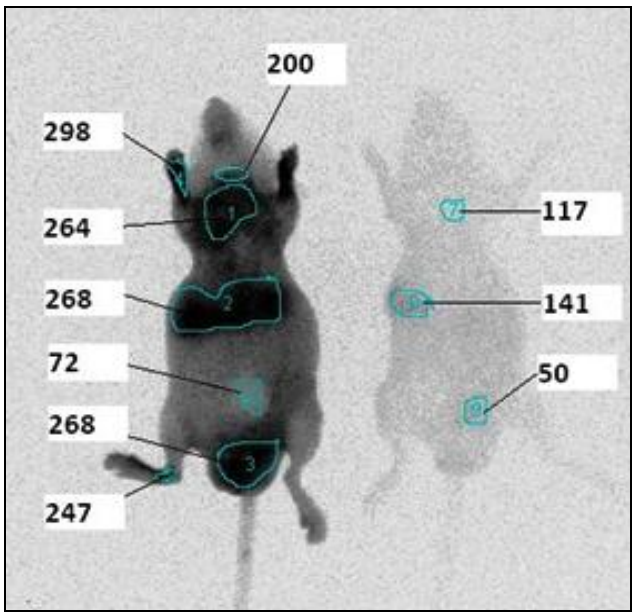

FIGURE 12: MOUSE INJECTED BY DiR LOADED PCLF2000 NPs SUSPENSION (A) AND DiR SOLUTION (B), INTO HEART, AFTER 72 HOURS

This study examined lymph node retention of NPs up to 72 hours, and showed that 200-300 nm PCLF NPs were significantly present at qualitatively consistent levels in lymph nodes at 24, 48 and 72 hours following a bolus intracardial injection of 20 µl of samples. Images of mice injected by formulations were quantitatively analyzed (**Figures 13-16**). For every organ of the mouse body, the mean intensity of light was measured which represented the concentration of the formulation in that organ.

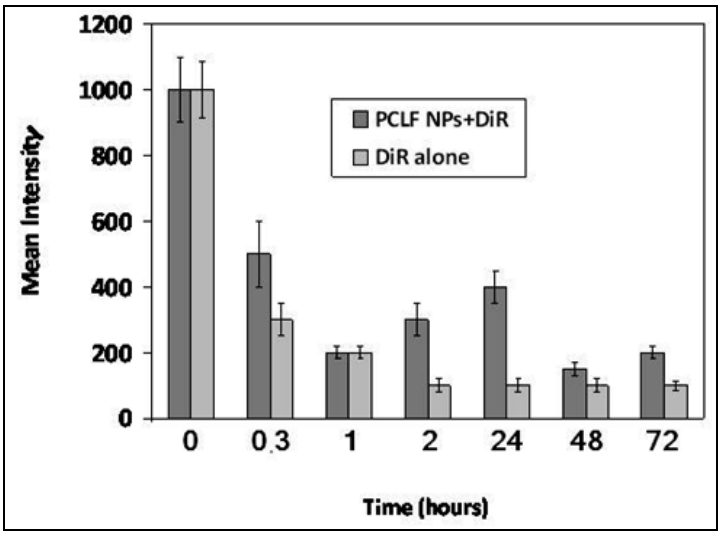

FIGURE 13: MEAN INTENSITY OF DiR FLUORESCENT IN HEART OF MOUSE INJECTED by DiR LOADED PCLF2000 NPs SUSPENSION AND DiR SOLUTION

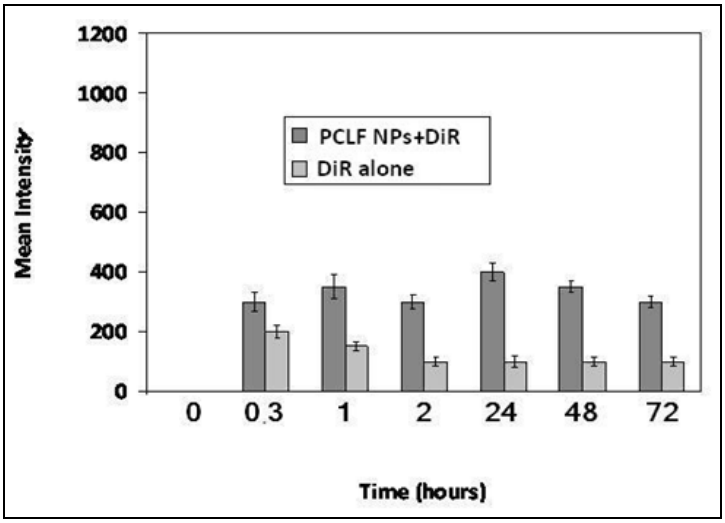

FIGURE 14: MEAN INTENSITY OF DiR FLUORESCENT IN LIVER AND SPLEEN OF MOUSE INJECTED BY DiR LOADED PCLF2000 NPs SUSPENSION AND DIR SOLUTION

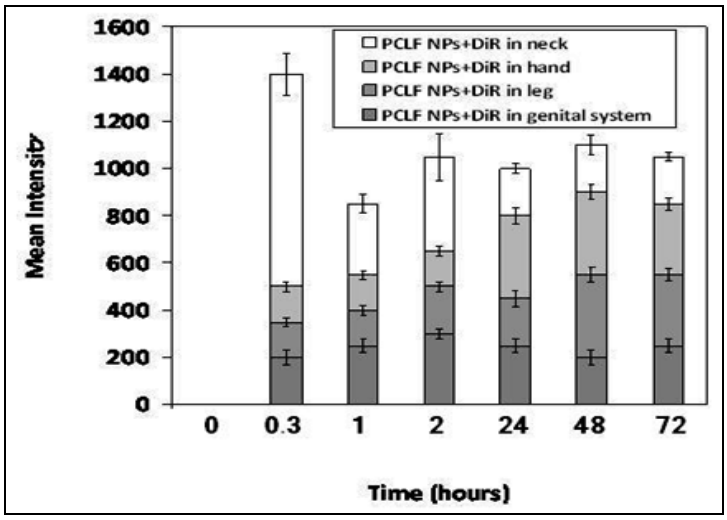

FIGURE 15: MEAN INTENSITY OF DiR FLUORESCENT IN NECK, HAND, LEG AND GENITAL SYSTEM OF MOUSE INJECTED BY DiR LOADED PCLF2000 NPs SUSPENSION AND DiR SOLUTION

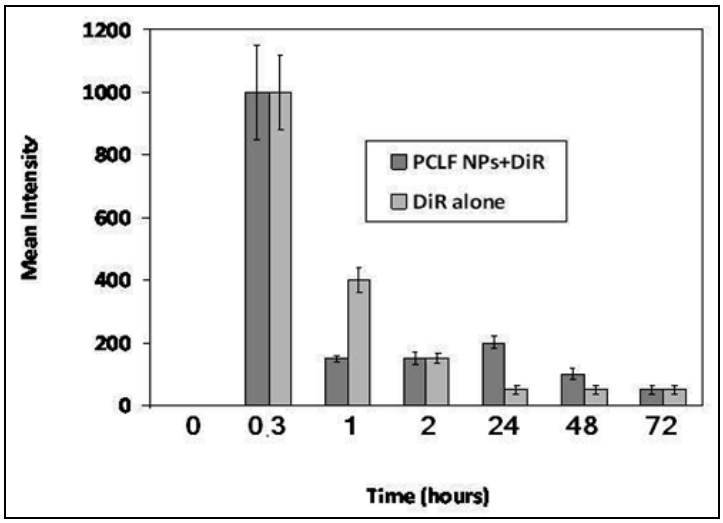

FIGURE 16: MEAN INTENSITY OF DiR FLUORESCENT IN BLADDER OF MOUSE INJECTED BY DiR LOADED PCLF2000 NPs SUSPENSION AND DIR SOLUTION

For instance, **figure 11** presents a comparison of the images of mice injected by the PCLF2000 NP suspension and DiR solution, after 3 days, which not only reveals the absence of DiR and the existence of the NPs in the body, but also shows the organs with high concentrations of the NPs, i.e. liver, spleen, hands, legs and genital system. Therefore, NP accumulation was occurred in these organs in contrast to the abdominal region which seemed empty and the bladder which did not show a dense color.

As it can be observed in the figures, DiR solution was eliminated from mouse body after about one hour post injection because the most density in the image was belonged to the bladder and not to the liver (**Figure 12 and 15**).

Suspensions of PCLF530 NPs, PCLF1250 NPs and PCLF2000 NPs were distributed in the body while just part of them was eliminated from the body (via urine) even after one to three days post injection (Figure 12 and 15).

CONCLUSION: PCLF copolymers with three different Mw (named PCLF530, PCLF1250 and PCLF2000) were used to prepare NPs. Dox loaded NPs showed controlled release duration of 4 days in PBS pH 5.8 with a higher maximum release compared to that in pH 7.4.

The cytotoxicity test of PCLF NPs on T47D, HT29 and 3T3NIH cells revealed that not only the empty PCLF NPs did not cause a considerable cytotoxicity and there was no significant difference between cytotoxicities of NPs of PCLF530, 1250 and 2000, but also the Dox loaded PCLF NPs showed great cytotoxicities which were in most cases equal to their corresponded concentration of Dox solutions.

Such results could additionally indicate that the NPs could be uptaken by these cells because the release duration of Dox from NPs was 4 days. Moreover, in cellular uptake test, the intracellular fluorescence of T47D cells, 48 hours after incubation with FITC loaded PCLF1250 NPs, proved the ability of these cells to uptake the PCLF NPs.

DiR fluorescent agent could load efficiently into the NPs and fortunately it did not show a fast release from NPs. Therefore, DiR could make NPs visible using *in vivo* imaging system. Comparison of images of the mice injected by DiR solution and DiR loaded NPs, revealed that in contrast to the DiR solution, DiR loaded NPs could remain in the mice body for a few days. Moreover, the NPs entered into the lymph node rich organs and were entrapped there after 1 day after injection. The injected mice did not show toxic symptoms or death following injection, confirming the cell cytotoxicity results.

ACKNOWLEDGEMENTS: The authors would like to express their sincere gratitude to Mehdi Gholami, Department of Pharmacology and Toxicology, and Hamed Hashemi, Faculty of pharmacy, Tehran University of Medical Sciences, for his assistance with the *in vivo* experiments.

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How to cite this article:
Shokri N, Azizi E, Javar HA, Sh. Fouladdel, Khalaj A and Dinarvand R: *In vitro* and *in vivo* Evaluation of Poly(Caprolactone Fumarate) Nanoparticles. *Int J Pharm Res Sci*. 3(9): 3106-3115.